A Micro Method for Simultaneous Determination of Galactosyltransferase and 5'-Nucleotidase Activities in Cell Fractions

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(Received 31 December 1974)

Galactosyltransferase and 5'-nucleotidase were assayed in the same reaction mixture, with ovalbumin as exogenous acceptor of [^{14}C]galactose and with [^3H]AMP as the substrate for the 5'-nucleotidase assay. The substrates and reaction products of either assay had no significant effect on the activity of the other enzyme.

Several investigators have described techniques for the assay of galactosyltransferase, an enzyme associated with glycoprotein biosynthesis and also thought to be a marker for the Golgi apparatus in various tissues. These assays consist of the incubation of radioactively labelled UDP-galactose with an exogenous acceptor in the presence of the tissue fraction to be assayed. Exogenous acceptors of galactose have included glycoproteins freed of galactose and sialic acid (McGuire *et al.*, 1965; Spiro & Spiro, 1968; Schachter *et al.*, 1970), *N*-acetylglucosamine (Fleischer *et al.*, 1969; Leelavathi *et al.*, 1970) and ovalbumin (Wagner & Cynkin, 1971). In general, these systems require preliminary preparation of substrate or lengthy work-up procedures, and some do not provide the opportunity for examination of the reaction mixtures after incubation to determine the presence of remaining substrate, substrate-breakdown products, or unexpected side products of the assay reaction.

Because of these limitations, a new galactosyltransferase assay has been developed. This technique is a modification of the method of Wagner & Cynkin (1971) involving the use of ovalbumin as an exogenous acceptor of [^{14}C]galactose from UDP-[^{14}C]galactose. The method uses paper chromatography to separate reaction components and products, with the advantage of permitting multiple assays, eliminating laborious work-up procedures, and providing information about the amount of substrate and degradation products present at the end of the incubation period. In addition, a micro method for the assay of 5'-nucleotidase activity has been coupled with the galactosyltransferase assay so that the activities of these two enzymes can be determined simultaneously with small amounts of a tissue sample. These new methods have been successfully used for the assay of Golgi and other cell fractions prepared from rat liver.

Materials and methods

UDP-galactose, Mes [2-(*N*-morpholino)ethane-sulphonic acid] and *N*-acetylglucosamine were purchased from Calbiochem, Los Angeles, Calif., U.S.A. Adenosine, AMP and ovalbumin were purchased from the Sigma Chemical Company, St. Louis, Mo., U.S.A. Triton X-100 was purchased from the Fisher Scientific Co., Fair Lawn, N.J., U.S.A. UDP-[^{14}C]galactose, [^{14}C]galactose and [^3H]AMP were obtained from the New England Nuclear Corp., Boston, Mass., U.S.A.

Golgi and other cell fractions were prepared from rat liver by the method of Leelavathi *et al.* (1970). All procedures were performed at 0-4°C. Minced rat liver was homogenized (Potter-Elvehjem) in 5 vol. of medium containing 0.5M-sucrose in 0.1M-potassium phosphate buffer, pH 6.65, plus 0.005M-MgCl₂ (all other sucrose solutions were prepared in this same buffer solution). The homogenate was then centrifuged at low speed (755g, 10min). Centrifugation of the resulting supernatant over 1 vol. of 1.3M-sucrose (105000g, 60min) formed a concentrated band ('membrane-felt') at the 0.5M/1.3M interface. This membrane-felt was aspirated, adjusted to 1.1M-sucrose and then placed on a discontinuous gradient consisting of equal volumes of 1.25M-sucrose, the 1.1M membrane-felt and 0.5M-sucrose. The gradient was centrifuged for 90min at 105000g to form a 'Golgi' band (0.5M/1.1M interface) and a 'smooth endoplasmic reticulum' band (1.1M/1.25M-interface). The bands were then aspirated and pelleted at 105000g for 90min. Samples of each pellet were used for determination of protein content (Lowry *et al.*, 1951; bovine serum albumin as standard), enzyme activity (described below) and for confirmation of morphology by electron microscopy on sections stained with uranyl acetate and lead citrate.

The complete incubation mixture for the simultaneous determination of galactosyltransferase and 5'-nucleotidase activities contained the following ingredients: Mes buffer (pH 6.5), 0.05M; MnCl_2 , 0.01M; Triton X-100, 0.1%; ovalbumin, 0.5mg; $[^3\text{H}]\text{AMP}$ (3.2×10^5 d.p.m.), 0.002M; UDP- $[^{14}\text{C}]\text{-galactose}$ (1.4×10^5 d.p.m.), 0.001M; plus 1 μl or 2 μl of tissue homogenate or subcellular fraction in a total volume of 25 μl . After 15 and 45 min of incubation at 37°C, 8 μl portions of each reaction mixture were removed and applied to a sheet (46cm $\times$ 57cm) of Whatman no. 1 filter paper for chromatography.

The chromatography was conducted as follows. Before samples of the reaction mixtures were applied to the paper, each origin was spotted with 0.5 μmol of EDTA and 0.05 μmol each of UDP-galactose, AMP and adenosine. (It was found that the MnCl_2 in the reaction mixtures caused erratic chromatography of UDP-galactose. The treatment of the chromatogram origins with an excess of EDTA to chelate the manganese permitted reliable chromatography of UDP-galactose.) The samples of the

reaction mixtures were then applied, allowed to dry, and the chromatograms were developed for 18h by descending chromatography in ethanol-1M-ammonium acetate, pH 7.8 (5:2, v/v).

The finished chromatograms could be examined for radioactivity with a strip scanner to obtain approximate measurement of $[^3\text{H}]\text{AMP}$, $[^3\text{H}]\text{-adenosine}$, $[^{14}\text{C}]\text{ovalbumin}$, UDP- $[^{14}\text{C}]\text{galactose}$ and degradation products such as $[^{14}\text{C}]\text{galactose}$. However, strip scanning was not utilized as a routine. AMP and adenosine spots were located by u.v. light, cut out, eluted with water, and samples of these eluates were placed in scintillation fluid (Beckman Ready-Solv VI) and assayed for radioactivity in a liquid-scintillation spectrometer (Beckman LS-230). Since $[^{14}\text{C}]\text{galactose}$ -labelled ovalbumin remains at or near the origin of the chromatograms, the origin plus the first 1 cm of the chromatogram was cut out, placed directly in scintillation fluid and assayed in the liquid-scintillation spectrometer. This direct assay of the origins was found to yield approx. 70% of the ^{14}C radioactivity that would have been observed if the origins had instead been eluted with proteolytic enzymes followed by an assay of these eluates.

For comparison with the ovalbumin-acceptor method, galactosyltransferase activity was also assayed with *N*-acetylglucosamine as the acceptor for $[^{14}\text{C}]\text{galactose}$ transfer (from UDP- $[^{14}\text{C}]\text{galactose}$) to form $[^{14}\text{C}]\text{galactose}$ -labelled *N*-acetyl-lactosamine (Leelavathi *et al.*, 1970).

Results and discussion

A typical chromatogram is shown in Fig. 1, illustrating the separation of the reaction components and products. Assay of galactosyltransferase by incorporation of $[^{14}\text{C}]\text{galactose}$ into ovalbumin is shown in Table 1, together with the parallel assay utilizing *N*-acetylglucosamine as galactose acceptor for comparison. Similar enrichment of galactosyltransferase activity in the Golgi fraction was demonstrated with both methods, and the findings were consistent with the galactosyl-

Fig. 1. Chromatogram of a representative assay illustrating separation of reaction components and products

(a) A strip scan for radioactivity and (b) the location of spots seen under u.v. light are shown.

Table 1. Galactosyltransferase and 5'-nucleotidase activities of cell fractions

See the text for details of assay conditions.

Fraction	10 ⁻³ $\times$ $[^{14}\text{C}]\text{Galactose}$ incorporated (d.p.m./45 min per mg of protein)		10 ⁻³ $\times$ $[^3\text{H}]\text{Adenosine}$ formed (d.p.m./45 min per mg of protein)
	Into ovalbumin	Into <i>N</i> -acetylglucosamine	
Homogenate	10	11	4700
755g supernatant	9	16	4600
Membrane-felt	26	32	3500
Golgi fraction	660	840	29000
Smooth endoplasmic reticulum	150	160	37000

transferase activity of liver Golgi reported by others (Fleischer *et al.*, 1969; Leelavathi *et al.*, 1970; Morré, 1971; Bergeron *et al.*, 1973). By our technique, the galactosyl-ovalbumin transferase activity of the Golgi fraction was approx. 150nmol of galactose transferred/h per mg of protein. The amount of [^{14}C]galactose incorporated into *N*-acetyl-lactosamine was similar. Results with 15 min and 45 min incubations indicated that the reactions were linear for the full 45 min incubation. Cell fractions could be repeatedly frozen and thawed or stored frozen for a minimum of several weeks with no appreciable change in galactosyl-ovalbumin transferase activity. This assay has been made as much as thirtyfold more sensitive by increasing the amount of UDP-[^{14}C]galactose to as high as 4×10^6 d.p.m. per incubation mixture while keeping the concentration of UDP-galactose constant. Under these more sensitive conditions, UDP-[^{14}C]galactose is still quantitatively separated from ovalbumin-[^{14}C]galactose. However, enough UDP-[^{14}C]galactose overlaps the AMP and adenosine spots so that the simultaneous assay of 5'-nucleotidase activity (see below) cannot be easily performed, without concomitantly increasing the specific radioactivity of the [^3H]AMP.

The 5'-nucleotidase assay was performed in the reaction mixtures as the galactosyl-ovalbumin transferase assay and is also shown in Table 1. The nucleotidase activity of the Golgi fraction was about six times higher than that of the homogenate, in agreement with other studies (Morré, 1971; Bergeron *et al.*, 1973) demonstrating a moderate but consistent enrichment in the 5'-nucleotidase activity of liver Golgi fractions. The observed formation of adenosine from AMP was approx. 5 $\mu\text{mol/h}$ per mg of protein for the Golgi fraction, a value also in agreement with other studies (Bergeron *et al.*, 1973). We also found that the 5'-nucleotidase had a broad pH optimum, ranging from pH 5.5 to 7.5. Thus, under the assay conditions used in this study (pH 6.5), near-maximal activity was present.

Since Mg^{2+} has generally been used by others (Emmelot & Bos, 1966; Morré, 1971; Bergeron *et al.*, 1973) in 5'-nucleotidase assays and we have used Mn^{2+} rather than Mg^{2+} , we also examined the effects of these bivalent cations on 5'-nucleotidase activities. Neither added Mg^{2+} (0.01–0.1 M) nor Mn^{2+} (0.001–0.01 M) had significant effects on this reaction. In

contrast, an absolute requirement of the galactosyl-ovalbumin transferase reaction for Mn^{2+} was observed, supporting the earlier observations made by Wagner & Cynkin (1971).

The presence of UDP-galactose did not significantly affect 5'-nucleotidase activity; neither AMP nor adenosine (0.002 M) + phosphate (0.002 M) had significant effect on galactosyltransferase activity.

The product of the addition of [^{14}C]galactose to ovalbumin has not been defined, although it probably is to *N*-acetylglucosamine residues in the glycoprotein. However, the striking similarity between galactosyl-ovalbumin transferase and the transfer to *N*-acetylglucosamine indicates that the ovalbumin-acceptor method is equally as useful as the latter system for detecting Golgi marker enzymes. Further, when the ovalbumin-acceptor method is coupled with the 5'-nucleotidase assay, there exists the additional advantage of simultaneous determination of two membrane-related enzyme activities in the same incubation mixture for a more controlled comparison. Since 20–40 assays can be conducted as a routine in a few hours of working time, the method combines speed with reliability plus the capability for determination of substrate breakdown.

This work was supported in part by U.S.P.H.S. Grant no. AM-08816, N.I.A.M.D.D.

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